Am. J. Trop. Med. Hyg., 58(6), 1998, pp. 812–815

A SINGLE TISSUE CULTURE SYSTEM FOR THE PROPAGATION OF THE AGENTS OF THE HUMAN EHRLICHIOSES

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Abstract. Two newly emergent human diseases found in the United States, human monocytotropic ehrlichiosis (HME) and human granulocytotropic ehrlichiosis (HGE), are caused by pathogens of the genus *Ehrlichia*. The causative agent of HGE can be propagated in HL-60 human promyelocytic leukemia cells. Herein, we report the development of a method to propagate *E. chaffeensis*, the causative agent of HME, in HL-60 cells, thus providing a common system for the study of both species. The continuous propagation of *E. chaffeensis* requires the induction of HL-60 differentiation along the monocytic pathway toward phenotypically mature macrophages by the addition of 25-OH vitamin D_3 to the growth medium.

Two species of the genus *Ehrlichia* are known to cause human disease in the United States. The first species, *E. chaffeensis*, was identified in 1991 and is associated with human monocytotropic ehrlichiosis.[1, 2] The second species, the causative agent of human granulocytotropic ehrlichiosis (HGE), is closely related to or identical with *E. equi*.[3–5] Each of these *Ehrlichia* species appears to be transmitted by ticks, albeit by different species of tick–*E. chaffeensis* by *Amblyomma americanum* and the HGE agent by *Ixodes scapularis.* While infections caused by these *Ehrlichia* species are treatable with tetracycline and its derivatives, they can cause live-threatening and even fatal disease if untreated.[3, 6–8] Thus, rapid recognition and identification of the pathogens are important in the diagnosis and treatment of human ehrlichioses.

Also important for the study of these emerging pathogens are techniques for their culture and propagation. *Ehrlichia chaffeensis* was first isolated and characterized after passage in DH82 cells, a continuous canine macrophage cell line that grows as a monolayer culture.[1] It has subsequently been propagated in a variety of monolayer cell lines of different tissue origins from different species.[9, 10] The agent of HGE and *E. equi* can be propagated in HL-60 cells, which grow in suspension culture.[11] The HL-60 cells are a human promyelocytic leukemia cell line that is capable of differentiating into either functional monocytes or granulocytes in the presence of appropriate differentiating agents.[12, 13] We have recently demonstrated that *E. equi* can be more efficiently propagated in HL-60 cells induced to differentiate down the granulocytic pathway than in undifferentiated HL-60 cells.[5]

For the purposes of further study into the mechanism of *Ehrlichia* entry and pathogenesis, and for comparisons between the two species, it would be useful to identify a single mammalian tissue culture system capable of sustaining the growth and proliferation of both species. We report here that *E. chaffeensis* can be propagated in HL-60 cells induced to differentiate down the monocytic pathway but does not propagate in undifferentiated or in granulocytically induced HL-60 cells. Thus, there now exists a single cell line that can be used to study both *Ehrlichia* species and that can be used to make comparisons that are not influenced by the use of different host cells grown with different media under different culture conditions.

MATERIALS AND METHODS

Biological materials. The HL-60 cells, a continuously growing, transformed suspension cell line,[14] was obtained from the laboratory of Dr. Alan C. Sartorelli at Yale University School of Medicine. Cells were maintained by twice weekly passage in a medium containing RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum, pyruvate, nonessential amino acids, and glutamine (RP-U). Monocytic differentiation was accomplished by the addition of 2 μM 25-OH vitamin D_3 to the medium (RP-VD); granulocytic differentiation was achieved by the addition of 1 μM retinoic acid (RP-RA).

Ehrlichia chaffeensis was propagated in DH82 cells as previously described.[1] Briefly, DH82 cells were maintained in monolayer culture in a medium containing minimum essential medium supplemented with 12.5% heat-inactivated fetal bovine serum and L-glutamine. Cultures were fed twice a week with fresh medium to maintain the monolayers. The percentage of DH82 cells infected with *E. chaffeensis* was determined by cytologic staining and light microscopic observation of morulae. When more than 25% of cells were infected, cultures were passaged by diluting resuspended infected cells with three times the number of cells from uninfected cultures and replating in fresh medium.

Transfer of *E. chaffeensis* to and propagation in HL-60 cells. Monolayer cultures of DH82 cells growing in 25-cm^2 tissue culture flasks in which 25–50% of the cells were infected with *E. chaffeensis* were used in attempts to transfer the pathogen to HL-60 cells. Supplemented minimal essential medium was removed and replaced with 12 ml of RP-U containing 2.5×10^6 undifferentiated HL-60 cells, 12 ml of RP-VD containing 5×10^6 HL-60 cells exposed to 25-OH vitamin D_3 for four days, or 12 ml of RP-RA containing 5 x 10^6 HL-60 cells exposed to retinoic acid for four days. Cocultures of HL-60, DH82, and *E. chaffeensis* were maintained for four days before 6 ml of suspension cells were removed into new 25-cm^2 tissue culture flasks containing 6 ml of fresh medium supplemented, where appropriate, with the inducing agent.

After four days further in culture, all putatively infected HL-60 cultures were passaged into 12.5-cm^2 tissue culture flasks. Cultures containing differentiated HL-60 cells were maintained by weekly passage in which 1.5×10^6 of the old cells were mixed with an equal number of uninfected but

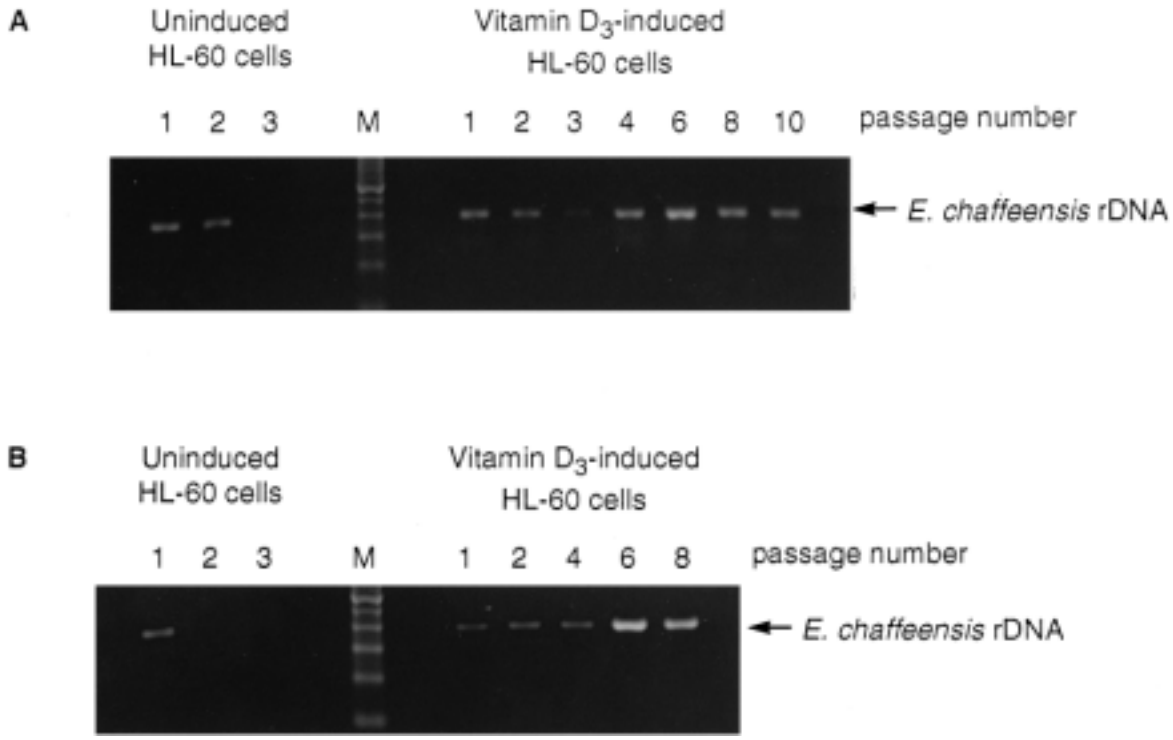

FIGURE 1. Polymerase chain reaction amplification of *Ehrlichia chaffeensis* rDNA in passages of uninduced and 25-OH vitamin D_3–induced HL-60 cells. Results in **A** and **B** are from two different sets of passages, each of which originated as a coculture of infected DH82 monolayer cells and uninfected HL-60 cells that had either been maintained uninduced or been induced to differentiate into monocytes by the addition of 25-OH vitamin D_3 to the growth medium. A 100-basepair ladder served as markers (lanes M).

differentiated HL-60 cells in a total of 6 ml of RP-VD or RP-RA as appropriate. Cultures containing undifferentiated HL-60 cells were maintained by alternating passage with subdivision. At each passage, 0.75×10^6 of the old cells were mixed with an equal number of uninfected, undifferentiated HL-60 cells in a total of 6 ml RP-U in 12.5-cm^2 tissue culture flasks. After three days, the culture was subdivided by adding a 1-ml aliquot of the culture to 5 ml of fresh RP-U medium. Four days thereafter, the cultures were passaged.

Detection of *E. chaffeensis* in HL-60 cells. The persistence of *E. chaffeensis* in DH82 monolayers or in HL-60 suspension cultures as they approached plateau phase with cell densities of 1.5×10^6 cells/ml was determined by specific amplification of the ehrlichial 16S rDNA. Bacterial DNA was extracted from infected tissue cells by a modification of the method of O'Neill and others.[15] Briefly, extraction started with 0.5 ml of HL-60 cells in suspension cell culture or the same volume of DH82 monolayer cells resuspended by scraping a confluent culture with a rubber policeman. By this method, approximately 750,000 HL-60 or 500,000 DH82 cells were collected. Cells were washed once with phosphate-buffered saline, the cell pellet was resuspended in 100 μl of a lysis buffer containing 150 mM NaCl, 10 mM Tris-HCI, pH 8.0, 1 mM EDTA, and 100 μg/ml of proteinase K, and the lysate was incubated for 1 hr at 55°C. The proteinase K was inactivated by incubation at 100°C for 10 min. A polymerase chain reaction (PCR) was run for 35 cycles using the equivalent of 10 μl of the initial cell culture (approximately 15,000 HL-60 or 10,000 DH82 cells) and *E. chaffeensis*-specific oligonucleotide primers.[16] Amplified DNA was separated from primers by electrophoresis on 2.5% agarose gels and visualized by staining with ethidium bromide.

Light microscopic detection of *Ehrlichia*-infected HL-60 cells was done by cytologic staining with Wright stain followed by Giemsa stain.

RESULTS

Attempts were made to transfer *E. chaffeensis* from DH82 cells growing in confluent monolayers in which at least 25% of the cells were infected to uninfected HL-60 cells by coculture with undifferentiated HL-60 cells, HL-60 cells induced with retinoic acid to undergo granulocytic differentiation, or HL-60 cells induced with 25-OH vitamin D_3 to undergo monocytic differentiation. In the first set of experiments, the HL-60 cells recovered from these cocultures were passaged to maintain their differentiated status. On two separate occasions, infected DH82 cells were cocultured with undifferentiated or 25-OH vitamin D_3–induced HL-60 cells, and *E. chaffeensis* DNA was recovered and amplified at the end of the first passage with each culture (Figure 1). On these two occasions, we maintained the cultures for 10 and nine passages, respectively. When infected cultures were maintained in RP-VD and passaged with addition of 25-OH vitamin D_3–induced HL-60 cells, *E. chaffeensis* DNA continued to be present. In contrast, all evidence of *E. chaffeensis* infection was lost in the undifferentiated cultures by the third passage. In one instance, no *E. chaffeensis* DNA was recovered and amplified after the second passage. The absence of morulae visible by light microscopy within the

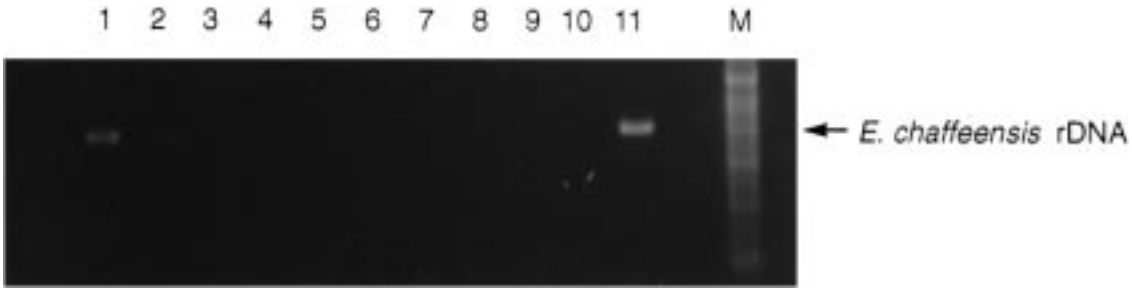

FIGURE 2. Polymerase chain reaction (PCR) amplification of *Ehrlichia chaffeensis* rDNA in HL-60 cells transferred between standard and differentiation-inducing growth media. Target DNA for the PCR in lanes 1–9 came from plateau phase cells passaged from cocultures containing uninduced HL-60 cells in standard medium. Uninduced HL-60 cells were first cocultured with infected DH82 cells and then transferred to a new flask with fresh medium (lane 1), transferred again into fresh medium (lane 2) or into medium containing 25-OH vitamin D_3 (lane 3). The cells were then passaged. Uninduced cells were either passaged with uninduced HL-60 cells in standard medium (lane 4) or with induced HL-60 cells into medium containing 25-OH vitamin D_3 (lane 5). Induced HL-60 cells were passaged with induced HL-60 cells into medium containing 25-OH vitamin D_3 (lane 6). Each of these three cultures were passaged once more with induced HL-60 cells into medium containing 25-OH vitamin D_3 (lanes 7–9). Target DNA for the PCR in lane 10 came from plateau phase uninduced HL-60 cells grown for three passages following the mixture of *E. chaffeensis*-infected, 25-OH vitamin D_3–induced HL-60 cells with uninduced HL-60 cells in standard medium. *Ehrlichia chaffeensis* DNA from DH82 cells served as a positive control (lane 11) and a 100-basepair ladder served as markers (lane M).

undifferentiated HL-60 cells confirmed the PCR data. When infected DH82 cells were cocultured with retinoic acid–induced HL-60 cells, no *E. chaffeensis* DNA was recovered and amplified after the second passage.

In a second set of experiments, we sought to determine the nature of the observed loss of *E. chaffeensis* from the uninduced cultures of HL-60 cells. We first attempted to infect undifferentiated HL-60 cells with *E. chaffeensis* by coculture with infected DH82 cells and then enhance ehrlichial propagation by inducing the differentiation of the infected HL-60 cells by growing them in RP-VD medium. A 6-ml aliquot of the uninduced suspension cells from the coculture was transferred to a new flask without the DH82 cell monolayer. After four days in culture, the ehrlichial DNA was still detectable by PCR (Figure 2, lane 1). Cells were then split; half remained in RP-U and the other half was induced by the addition of 25-OH vitamin D_3 to a final concentration of 2 μM. In both cases, *E. chaffeensis*-specific PCR products were undetectable after seven days (Figure 2, lanes 2 and 3, respectively). The cells from the uninduced and induced cultures were passaged. Uninduced cells were combined with logarithmically growing, uninduced HL-60 cells in RP-U or with 25-OH vitamin D_3–induced HL-60 cells in RP-VD. Induced cells were combined only with 25-OH vitamin D_3–induced HL-60 cells in RP-VD. As the cultures approached plateau phase, cells were harvested and the presence of ehrlichial DNA was determined by PCR (Figure 2, lanes 4-6). No PCR products were detectable. At this point, cytologic staining confirmed the absence of ehrlichial morulae. Cells were passaged once again. This time, all cultures were passaged with induced cells in RP-VD and tested for the presence of ehrlichial DNA upon approach to plateau densities. All evidence of *E. chaffeensis* infection was lost by this passage, despite the change to a more supportive environment and addition of infectable, 25-OH vitamin D_3–induced HL-60 cells (Figure 2, lanes 7–9).

We also sought to determine whether the persistent infection of 25-OH vitamin D_3–induced HL-60 cells would result in an adaptation that might permit the bacteria to propagate in undifferentiated HL-60 cells. After five passages in RP-VD, infected differentiated HL-60 were passaged into RP-U and mixed with undifferentiated HL-60 cells. Thereafter, these cultures were continued by passage into RP-U and addition of undifferentiated HL-60 cells. After three passages with uninduced HL-60 cells in RP-U, the cells were harvested and analyzed by PCR for the presence of ehrlichial DNA. All evidence of *E. chaffeensis* infection was lost in the undifferentiated cultures by this passage (Figure 2, lane 10). In contrast, *E. chaffeensis* maintained in the 25-OH vitamin D_3–induced HL-60 cells continued to be detectable by PCR (Figure 1B, passage number 8).

DISCUSSION

This report represents an attempt to unify the study of the agents of the human ehrlichioses by providing a single tissue culture system for the study of the HGE agent and *E. chaffeensis.* This work was predicated on the ability of HL-60 cells to differentiate into either macrophages or granulocytes, and on the demonstration that the HGE agent can be propagated in HL-60 cells whether continuously replicating, uninduced or terminally differentiated down the granulocytic pathway by retinoic acid.[5, 11] The work reported herein revealed that unlike the agent of HGE, *E. chaffeensis* could not be continuously propagated in uninduced HL-60 cells. The detection of ehrlichial 16S rDNA by PCR in the first passages in these cultures was probably the result of the shedding of infected DH82 cells from the monolayer into the growth medium. The infected DH82 cells do not proliferate in suspension and are diluted out as the cultures are passaged. This supposition is supported by the failure to observe ehrlichial morulae in undifferentiated HL-60 cells.

Although *E. chaffeensis* could not be continuously propagated in uninduced HL-60 cells, it could grow and reinfect HL-60 cells terminally differentiated along the monocytic pathway by the addition of 25-OH vitamin D_3 to the growth medium. Thus, one can envision a simple strategy to culture a pathogen from the blood of an individual suspected to have one of the known human ehrlichioses. By coculturing white blood cells with either granulocytically or monocytically differentiated HL-60 cells, it can be determined with which species the individual is infected. Because of the rapid dis-

appearance of *E. chaffeensis* from granulocytically induced cells (and the equally rapid disappearance of *E. equi* from monocytically induced cells[5]), the two species can be distinguished while simultaneously propagated for further study.

The failure of *E. chaffeensis* to grow in uninduced cells suggests that a feature of the mature macrophages that is missing from the continuously proliferating promyelocytic precursor HL-60 cells is required for *E. chaffeensis* entry into or proliferation within these cells. The results from the second set of experiments–in which transfer of uninduced, cocultured cells to differentiating medium fails to promote *E. chaffeensis* proliferation–and the failure to observe morulae in uninduced HL-60 cells are consistent with the former hypothesis, that the induction of differentiation is necessary for the entry of the pathogen into HL-60 cells. Even if the failure of *E. chaffeensis* to grow in the uninduced cells causes one to conclude that the two species of *Ehrlichia* cannot be grown in exactly the same cells, one important result of this study is to specify a narrow range of inducible gene products in HL-60 cells that can be scrutinized to learn more about the pathogenesis of *E. chaffeensis.*

Financial support: This work was supported by the G. Harold and Lelia Y. Mathers Charitable Foundation and the Centers for Disease Control and Prevention Emerging Infections Program (U50/CCU111188-01). Detra Tisdale was supported by the Summer Biomedical Research Training Program for Minority Students (T35-HL07722-05) from the National Heart, Lung, and Blood Institute.

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